Electrophoretic variation in the Green and Golden Bell Frog *Litoria aurea*

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ABSTRACT

Protein electrophoresis was used to investigate the genetic structure of populations of the Green and Golden Bell Frog *Litoria aurea* in the Sydney area. The investigation provided the following answers to three main questions. 1. Has habitat reduction and fragmentation reduced genetic variability in the Sydney area? Possibly yes, but not drastically to date. 2. Is population differentiation in the area due to the loss of genetic variability following from restrictions on gene flow? Not solely, as there exist differences in the frequency of the G6PD A allozyme in samples from the east of Sydney and in samples from the west. 3. Do there exist variants in Sydney samples which, not being found elsewhere in the species' range, emphasize the importance of efforts to conserve populations in the area? There are large numbers of alleles restricted to a single sample in this study (including eight in the Sydney area) which suggest that variability in *L. aurea* should be understood in terms of regional differentiation.

INTRODUCTION

The electrophoretic investigations of the Green and Golden Bell Frog, *Litoria aurea* detailed in this paper were principally concerned with assessing the frog's population genetic structure in the Sydney region. In particular, the investigations were aimed at determining the answers to three sets of questions.

1. Has habitat reduction and fragmentation in the course of urbanization reduced levels of genetic variability within local isolates or within the area as a whole? If it has done so then the likelihood is high that these populations would become extinct in the intermediate term because of the deleterious consequences of inbreeding on general fitness and disease resistance (Allendorf 1983; Vrijenhoek 1989; Woodruff 1989). In such a situation, the case for active intervention in the gene pool of these populations depends on the answers to the next sets of questions.
2. Is the differentiation of local populations within the region ascribable to the loss of genetic variability following from restrictions on gene flow? If so, then introduction of genetic material from other populations in the area might increase the chances of longer term survival of the species. If not, the introduction might disrupt gene combinations which have evolved in response to very localized conditions, with a consequent short term reduction in fitness which may prejudice population survival.
3. Do there exist variants in Sydney populations which are not found elsewhere in the species' range? If such variants are found then significant efforts, perhaps including genetically-monitored captive breeding programmes, should be made to ensure the conservation of the species' diversity.

A subsidiary aim of this work is to establish whether electrophoretic methods can resolve the problem of discriminating between the tadpoles of various *Litoria* species which are not definitely distinguishable by morphological criteria (A. Greer, pers. comm.). If this approach were successful it would permit rapid determination of whether the endangered *L. aurea* is present at a site or whether a tadpole population is composed of species which are not currently considered threatened, such as *L. peroni, L. tyleri* or *L. dentata.*

METHODS

Metamorphlings or tadpoles of *L. aurea* were collected from the following localities. Two sites lying on either side of Haslam's Creek were sampled within the Homebush Bay area; one from the Newington Armaments Depot and the other from the "brickpit". The other Sydney area sites were the G.I.O. property at Rosebery, Sydney Water's brickpit at the corner of Juno Pde and Punchbowl Rd, South Strathfield and Sir Joseph Banks Drive, Kurnell. A sample was taken just west of Nowra, near the Shoalhaven River to act as a reference for genetic variation in a large, relatively undisturbed population. Because of the small population sizes and geographic area of most sample localities, it seems reasonable to assume that many of individuals from particular sites were sibs. Small samples were also available from Mt Aoupinie and Mt Koghis in New Caledonia where *L. aurea* has been introduced. Tadpoles were identified by comparison of their electrophoretic phenotypes with adults whose specific status was known. *L. peroni* tadpoles were found in the Lungfish breeding pond at Macquarie University and Kirby Rd, Dundas. *L. dentata* tadpoles were taken from the Newington Armaments Depot and *L. tyleri* tadpoles from The Chase, Valley Heights, Blue Mountains.

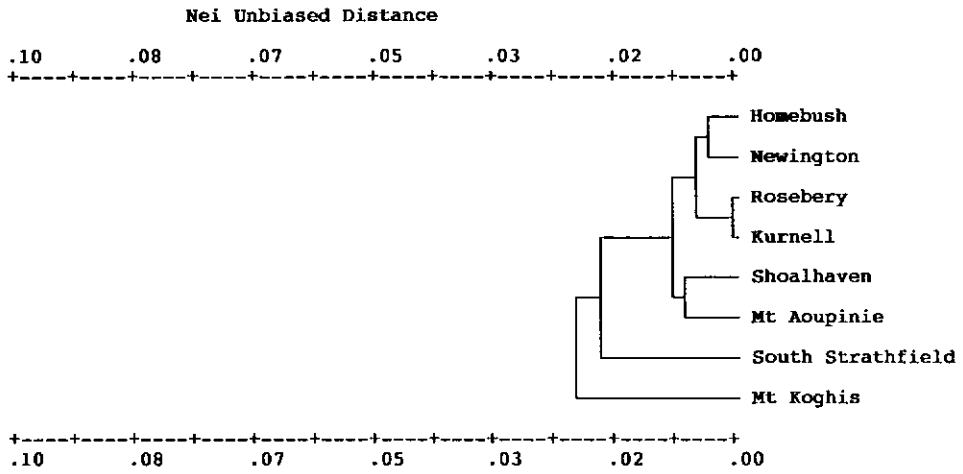

Fig. 1. Phenogram of relationships between *Litoria aurea* populations based on an UPGMA clustering of Nei's unbiased genetic distance.

Tissues were taken from specimens after they had been sacrificed by chlorotone immersion. Liver was taken from metamorphlings and anterior portions of the head from older tadpoles. The whole body was used for tadpoles sacrificed less than one week after hatching from egg masses. Protein electrophoresis of tissue homogenates was performed on Titan III cellulose acetate gels using standard procedures (Richardson *et al.* 1986; Manchenko 1994) using the buffers and stains detailed in Colgan *et al.* (1993). Allozymes are designated alphabetically in order of their anodal mobility. Loci are given numbers to indicate their position, "1" being that which migrates most towards the anode. For *Gpd* and *Pgk*, tadpoles had cathodal isozymes not seen in other stages. Population heterozygosity, F_{ST} statistics and genetic distances between populations were calculated with the assistance of the BIOSYS–1 programme package (Swofford and Selander 1981), which was also used to calculate phenograms. Estimates of the product *(Nm)* of population size and migration rate between populations (which includes historic events as well as current levels) were made using the F_{ST} statistics approach of Wright (1951) and the private alleles ($\bar{p}$ (1)) approach of Slatkin (1985). For the F_{ST} approach, *Nm* is calculated from the relation:

$$F_{ST}\,(1 + 4Nm)^{-1}$$

For the $\bar{p}(1)$ approach, *Nm* is calculated from the relation:

$$\mathrm{Log}_{10}(\bar{p}(1)) \approx a\ \mathrm{Log}_{10}(Nm) + b$$

where *a* and *b* are constants depending on sample size. Here, values for a sample size of 25 are used as this is the set calculated by Slatkin and Barton (1989) which is closest to the average sample size of the Australian populations in this study.

RESULTS

The electrophoretic data are summarized in three tables: Table 1 gives details of the allozymic frequencies at each scored locus in all populations; Table 2 presents intra-population variability statistics for *L. aurea* populations; and Table 3 gives two alternative measures of the genetic distance between pairs of populations.

Genetic distances between populations, including those from New Caledonia, are low using either measure (Fig. 1). Despite the low overall distances, there are indications that there are significant genetic differences between populations, with most having at least one allele which is not found elsewhere (private alleles). The Shoalhaven population has seven such alleles. The highest number of private alleles (five) in the Sydney area is found in the Newington population. In total, there are nine alleles which are found in the Sydney area which are not seen in the Shoalhaven. Only one of these is seen in New Caledonia.

The overall average F_{ST} value including all variable loci is 0.261 giving an estimated migration rate of $Nm = 0.71$. The overall average frequency for private alleles $(\bar{p}(1))$ is 0.130 giving an estimated migration rate of 0.43. When Shoalhaven and the Sydney area samples are considered, the *Nm* estimated from F_{ST} is 1.41 and that from $\bar{p}(1)$ is 0.42. When only the Sydney area populations are included, the average F_{ST} value is 0.155 giving $Nm \approx 1.36$ and $\bar{p}(1) = 0.158$, estimating *Nm* as 0.31. The private alleles estimates of migration may be somewhat inflated by the high frequency of the HBD2 A allozyme in the South Strathfield population. If this particular allozyme is excluded from the analyses, the estimates of *Nm* for the three sets of populations considered above are 0.75, 0.76 and 0.89. These are still very low but are more concordant with the F_{ST} estimates.

Table 1. Allozyme Frequencies in *Litoria aurea* and related species. The allozymes are designated alphabetically in order of their mobility. Loci are given numbers to indicate their position, "1" being the fastest. Locus abbreviations are generally standard, except that "PVL", "PLGG" and "PPP" are peptidases identified using, respectively, valine-leucine, leucine-glycine-glycine and phenylalanine-proline as substrates. Numbers in the same row as locus designations indicate sample sizes.

	Species and population										
	Litoria aurea								*Litoria*		
Locus	Homebush	Shoalhaven	Rosebery	Newington	Kurnell	Strathfield	Mt Aoupinie	Mt Koghis	*peroni*	*tyleri*	*dentata*
Acon	39	28	34	12	1			4	2	1	1
A	1.000	1.000	1.000	1.000	1.000			1.000			1.000
B									1.000	1.000	
Ak-1	39	10	34								
A	1.000	1.000	1.000								
Ak-2	39	10	34								
A	1.000	1.000	1.000								
Ak-3	39	10	34								
A	1.000	1.000	1.000								
Ak-4	35	10	33								
A	.271	.450	.227								
B	.729	.500	.773								
C		.050									
Ald	39	10	33								
A	1.000	1.000	1.000								
Aat1	39	26	35	12	11	4	1	3	5		3
A											1.000
B	1.000	.942	1.000	1.000	1.000	.875	1.000	1.000	1.000		
C		.058				.125					
Aat2	39	26	35	12	11	4	1	3	5		3
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000		
B											1.000
Enol	39	10	34								
A	1.000	1.000	1.000								
Est1	39	10	34								
A	1.000	1.000	1.000								
Est2	39	9	34								
A	1.000	1.000	1.000								
Fbp	39	16	35	12	8	4	1	3	3		3
A									1.000		
B	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000			1.000
Fh1	39	12	35	11	8	4		3	4		
A									1.000		
B	1.000	.917	1.000	1.000	1.000	1.000		1.000			
C		.083									
Fh2	39	16	35	12	8	4		3	4		
A									1.000		
B	1.000	.688	1.000	1.000	1.000	1.000		1.000			
C		.313									
G6pd	39	31	35	12	11	4	1	5	6	1	3
A											1.000
B			.286	.042	.409			.100			
C	1.000	1.000	.714	.958	.591	1.000	1.000	.900	1.000	1.000	
Gpi	39	31	34	12	11	4	1	5	6	1	3
A									1.000	1.000	1.000
B	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000			
Bgal	39	10	34								
A	1.000	1.000	1.000								
Ga3pdh	39	30	35	10	10	4	1	2	6	1	1
A										1.000	
B									.833		
C	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.167		1.000
Gpd	39	13	35	11	10	4	1	5	4		3
A	.051		.043								
B	.949	1.000	.957	1.000	1.000	1.000	1.000	1.000	1.000		1.000
Gpt	37	10	34								
A	1.000	1.000	1.000								
Idh1	39	15	35	12	8	4		1	4		
A	1.000	1.000	1.000	1.000	1.000	1.000		1.000			
B									1.000		
Idh2	39	25	35	12	11	4	1	4	5		3
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000		1.000
Hbd1	38	13	34	9	9	4	1	3	4		
A	.868	.846	.912	.944	.889	1.000	1.000	1.000			
B									1.000		
C	.132	.154	.088	.056	.111						

Table 1 — continued.

	Species and population										
	Litoria aurea								*Litoria*		
Locus	Homebush	Shoalhaven	Rosebery	Newington	Kurnell	Strathfield	Mt Aoupinie	Mt Koghis	*peroni*	*tyleri*	*dentata*
Hbd2	38	12	34	12	10	3	1	3	3		
A						.667					
B	1.000	1.000	1.000	1.000	1.000	.333	1.000	1.000			
C									1.000		
Ldh1	39	30	35	12	10	4	1	5	6	1	3
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000			
B									1.000	1.000	
C											1.000
Ldh2	39	30	35	12	11	4	1	5	6	1	3
A								.100			
B	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.900	1.000	1.000	1.000
Mdh1	39	28	35	12	11	4	1	5	6	1	3
A				.042					.083		
B	1.000	1.000	1.000	.958	1.000	1.000	1.000	1.000	.917	1.000	1.000
Mdh2	39	28	35	12	11	3	1	5	6	1	3
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000			
B									1.000	1.000	1.000
Me1	39	25	34	9	11	4	1	3	6	1	3
A	1.000	.980	1.000	1.000	1.000	1.000	1.000	1.000			
B		.020									.167
C											.833
D										1.000	
E									1.000		
Me2	39	30	34	12	11	4	1	5	6	1	3
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	
B											1.000
Mpi	39	31	35	12	11	4	1	5	6	1	3
A											1.000
B	1.000	.984	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	
C		.016									
PVL	39	30	34	9	11	4	1	5	6	1	3
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000			
B									1.000	1.000	
C											1.000
PLGG	39	13	32	5	10	4	1	3	4		3
A	.308	.192	.063								
B	.692	.769	.938	1.000	1.000	1.000	1.000	1.000			
C		.038									
D									1.000	1.000	
PPP	39	14	34	10							
A	1.000	1.000	1.000	.800							
B				.200							
Pgk	39	13	34	9			1				3
A											1.000
B	1.000	.961	1.000	1.000			1.000				
C		.038									
Pgm	39	31	34	12	11	4	1	5	6	1	3
A	1.000	.984	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B		.016									
6Pgd	26	28	26	12	11	4	1	5	6	1	3
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000			1.000
B									.750	1.000	
C									.250		
Sdh1	39	13	34	10	10	4		2	4		
A					.050				1.000		
B	1.000	1.000	1.000	.950	.950	1.000		1.000			
C				.050							
Sdh2	39	13	32	11	10	4		2	4		
A				.045					1.000		
B	.346	.231	.469	.364	.500	.125		1.000			
C	.654	.769	.531	.591	.500	.875	1.000				
Sod	39	10	34	4							
A	1.000	1.000	1.000	1.000							
Tpi	39	31	34	12	11	4	1	5	6	1	3
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Xdh	37	14	32	10	11	4	1	3	4		3
A									1.000		
B	.027	.107	.156								
C	.973	.893	.844	.950	1.000	1.000	1.000	1.000			1.000
D				.050							

Table 2. Summary measures of variability in populations of *Litoria aurea*. Standard errors of the measures are given in parentheses. A locus was considered polymorphic if more than one allele was detected in the sample. The expected heterozygosity is the unbiased Hardy-Weinberg value.

Population	Mean sample size per locus	Mean No. of alleles per locus	Percentage of loci polymorphic	Mean heterozygosity	
				Direct-count	HdyWbg expected
1. Homebush	38.4	1.2	16.7	.023	.042
	(.4)	(.1)		(.013)	(.022)
2. Shoalhaven	22.7	1.4	36.7	.027	.079
	(1.4)	(.1)		(.013)	(.025)
3. Rosebery	34.0	1.2	20.0	.023	.052
	(.3)	(.1)		(.011)	(.023)
4. Newington	10.9	1.2	20.0	.022	.034
	(.4)	(.1)		(.009)	(.019)
5. Kurnell	9.6	1.1	13.3	.026	.045
	(.5)	(.1)		(.016)	(.025)
6. South Strathfield	3.7	1.1	10.0	.017	.034
	(.1)	(.1)		(.012)	(.021)
7. Mt Aoupinie	1.0	1.0	.0	.000	.000
	(.0)	(.0)		(.000)	(.000)
8. Mt Koghis	3.7	1.1	6.7	.013	.013
	(.2)	(.0)		(.009)	(.009)

Table 3. Genetic distances between populations of *Litoria aurea*. The distances below the diagonal are values of Nei's unbiased genetic distance and those above are values of Wright's modified Rogers distance.

Population	1	2	3	4	5	6	7	8
1. Homebush	*	.075	.076	.062	.099	.144	.089	.137
2. Shoalhaven	.004	*	.101	.089	.122	.151	.096	.167
3. Rosebery	.005	.009	*	.057	.041	.153	.106	.110
4. Newington	.003	.006	.002	*	.073	.134	.074	.116
5. Kurnell	.009	.013	.000	.004	*	.162	.120	.111
6. South Strathfield	.019	.020	.021	.015	.024	*	.126	.204
7. Mt Aoupinie	.008	.007	.011	.005	.013	.014	*	.184
8. Mt Koghis	.018	.027	.011	.012	.011	.040	.034	*

The percentage of loci with more than one allele, and observed and expected heterozygosities in the Shoalhaven population are higher than any other sample of *L. aurea* (Table 2), even though the sample sizes at this populations are smaller than those taken at the Rosebery or Homebush sites. The differences in these intra-population measures are not, however, significant. The low proportions of polymorphic loci and low heterozygosity levels at Kurnell, South Strathfield, Mt Koghis and Mt Aoupinie are at least partly due to the small sample sizes from these localities.

The difference between samples in glucose-6-phosphate dehydrogenase allozymic frequencies is notable. A faster allozyme attains a frequency of 29% at Rosebery and 40% at Kurnell but is found, where present at all, in very low frequencies in western Sydney and Shoalhaven samples. It does occur on New Caledonia, with one heterozygote being observed at Mt Aoupinie.

The electrophoretic results show clear distinctions between all four studied *Litoria* species. Comparison of the electrophoretic phenotypes of tadpoles with adults of known identity revealed that there were no developmental differences in the great majority of enzyme systems, with *Pgk* and *Gpd* notable exceptions because they do have tadpole-specific isoenzymes. The variants which are seen in tadpoles for these enzymes but not in other stages may represent the products of stage-specific loci. The tadpole data for *Pgk* and *Gpd* are not included in frequency calculations. Tadpoles which were found by electrophoretic comparison with known adults to belong to *L. aurea, L. peroni, L. tyleri* and *L. dentata* (frozen less than one week after hatching) were available for study. Tadpoles of any of the four species are readily distinguished electrophoretically from those of other species. The *Aat, Gpi, Ldh, Mdh* and *Me* systems are reliable and relatively cheap for this purpose.

DISCUSSION

Genetic distances between samples are in the lower end of the range reported for conspecific frog populations (Case *et al.* 1975; Case 1978; Feder 1979; Ralin *et al.* 1983; Odendaal and Bull 1983; Barendse 1984; Titus *et al.* 1989). Estimates of migration rates from both F_{ST} and $\hat{p}(1)$ approaches are somewhat higher than the average in amphibians (Ward *et al.* 1992). Nor are there any instances where an allele is at a frequency of 100% in one sample but absent from another sample ("fixed differences").

These results suggest that there are no specific or sub-specific taxonomic divisions within our collections of *Litoria aurea*.

Heterozygosity levels in *L. aurea* populations are towards the lower end of the range reported of populations in frog species (Case *et al.* 1975; Feder 1979; Hayes and Miyamoto 1984; Zeyl 1993) and are significantly lower than the average found for amphibians (Ward *et al.* 1992). There appears to have been some lowering of intra-populational polymorphism and heterozygosity in populations from the Sydney area when compared to the Shoalhaven sample. The maximum percentage of polymorphic loci in samples from Sydney is 20, whereas the Shoalhaven sample has variation at 36.7% of loci. There is a similar disparity in expected heterozygosity. Neither of these comparisons are statistically significant but do, in conjunction with the generally low variability in *L. aurea*, present grounds for concern regarding loss of genetic variation in the Sydney region. If this is explicable as an effect of the population fragmentation and habitat size reduction which has taken place in Sydney then continuation of these processes will most likely lead to further losses of variability in local areas, with low frequency private alleles particularly at risk. As discussed in the next paragraph, this is not, however, an immediately compelling argument for wholesale translocation of individuals.

The fact that relatively close sets of populations (Rosebery and Kurnell versus other Sydney area samples) can exhibit such marked frequency differences as found at the *G6pd* locus necessitates caution when evaluating populations for destruction, relocation or admixture. It is unlikely that the low frequency of the G6PD A allozyme in western Sydney (Homebush and South Strathfield) populations is due to recent inbreeding. More likely are two other possibilities: (1) The allele has attained a higher frequency in the east due to some selective advantage; or (2) the regions have been sufficiently genetically-isolated for sufficient periods of time for differentiation to occur by drift. In either case, the artificial introduction of this allele (and the suite of other, unstudied alleles that it represents) into the western region might not increase the probability of that population's longer term survival. If the former possibility were correct, the chances of survival might even be reduced through outbreeding depression. Translocations in *L. aurea* might be considered for purposes of overcoming inbreeding depression or for re-establishing populations. The current results suggest that great caution should be exercised when contemplating translocations between populations (or areas) which are separated by more than a few tens of kilometres.

There is some evidence that populations in the Sydney area are sufficiently genetically distinct to warrant efforts for their preservation. There are no fixed electrophoretic differences between populations which might suggest that *L. aurea* in the area constitute multiple "Evolutionary Significant Units" *sensu* Moritz (1994). However, Moritz (1994) was at pains to point out that such units are relevant to the longer term conservation of the evolutionary potential of a species. For short and intermediate term conservation he suggested that attention be concentrated on "Management Units" which are "recognized as populations with significant divergence of allele frequencies at nuclear or mitochondrial loci, regardless of the phylogenetic distinctiveness of the alleles" (Moritz 1994). It seems probable that the *L. aurea* populations of the Sydney area comprise multiple Management Units defined by differences in the G6PD A allozyme frequencies. There are, additionally, eight other alleles which are observed in the Sydney area but not in the Shoalhaven sample. Although the New Caledonian samples are small, the fact that there are eleven allozymes in the Sydney samples that have not been found on the island, supports the concept that regional populations in *L. aurea* have notable suites of private alleles.

The large number of private alleles is worthy of further comment. Most populations, including Mt Koghis, have at least one such allele. Shoalhaven has the most, as might be expected from its distance from the other populations. However, Newington, which is separated from its nearest neighbour by less than 2 km, has five private alleles. These patterns suggest that *L. aurea* populations are recruited sporadically from a regional pool. By chance, and due probably to founder effects, only some of the low frequency alleles in this pool become represented at any given site. This has probably been the pattern of recruitment in this species during much of its evolution. Consequently, admixture of closely neighbouring populations with distinct suites of private alleles should not adversely affect longer term survival prospects.

The ease of using electrophoresis to distinguish tadpoles of different species suggests that it would be a rapid and reliable survey method for establishing the presence or absence of a nominated species at a particular site. The technique is applicable to tadpoles of all ages, including those less than a week old.

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APPENDIX

Material examined

The following "NR" numbers refer to specimen designations in the Australian Museum's frozen tissue catalogue for Herpetology. Not all specimens were examined for all loci.

Litoria aurea	
Homebush:	NR 1951–1990, 1992, 1993
Shoalhaven:	NR 2141–2149, 2229, 2735, 2736, 2738, 2240, 2244, 2246, 2247, 2249, 2253–2256.
Rosebery:	NR 2152–2186
Newington:	NR 2600–2602, 2616–2622, 2742, 2743
Kurnell:	NR 2150, 3192–3194, 3196–3201, 3207
South Strathfield:	NR 3215–3218
Mt Aoupinie:	NR 2890
Mt Koghis:	NR 3109–3113
L. peroni:	NR 2151, 2745, 3203–3206
L. tyleri:	NR 2738
L. dentata:	NR 3269, 3280 (two subsamples)